IJLST

ISSN: 0974-5335
IJLST (2016), 9(3):28-35

Erythrocyte Membrane and Hemolysis: Effects of Natural Products

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ABSTRACT:
The membrane bilayer and the network of membrane-associated proteins together regulate the characteristic shape and elastic properties of red blood cells (RBC). Hemolysis is the dissolution of red blood corpuscles with liberation of their hemoglobin. Incubated osmotic fragility testing is considered the gold standard in diagnosing the hematologic system in a patient with Coombs-negative spherocytic hemolytic anemia, particularly one of Northern European descent or with a positive family history of undiagnosed anemia. The use of natural products as medicine has increased in all over the world, and popular interest in complementary and alternative medicine in Brazil is also as large as it is in other countries especially with regard to phytotherapy, homeopathy and acupuncture. In this review are show the effects of natural products on osmotic fragility of erythrocytes due to alter the physic-chemical properties of the erythrocyte biomembrane.
Keywords: Biomembrane; Hemolysis; Osmotic fragility; Natural products; Biological effects.

INTRODUCTION

The membrane bilayer and the network of membrane-associated proteins together regulate the characteristic shape and elastic properties of red blood cells (RBC) [1]. When membrane skeletons are prepared in the presence of a high concentration of monovalent salt, the core of the membrane skeleton consists of spectrin, actin, protein 4.1, and dematin [2]. Spectrin is the most abundant protein in the membrane skeleton and plays a critical role in the maintenance of erythrocyte shape and membrane properties [1, 2, 3]. The mechanism by which the head region of spectrin binds to the membrane via ankyrin and band 3 has been relatively well characterized [3].

Membrane loss is due to defects in one of several membrane proteins, including ankyrin, band 3, α spectrin, βspectrin, and protein 4.2. Erythrocyte membranes from Hereditary Spherocytosis (HS) patients demonstrate qualitative and/or quantitative abnormalities of these proteins, most commonly combined spectrin and ankyrin deficiency, followed by band 3 deficiency, isolated spectrin deficiency, and protein 4.2 deficiencies [4].

Hemolytic anemia due to abnormalities of the erythrocyte membrane comprises an important group of inherited disorders. These include HS [4], and other syndromes [5] like hereditary elliptocytosis (HE), hereditary pyropoikilocytosis (HPP), and the hereditary stomatocytosis (HSt) syndromes. These disorders are characterized by clinical and laboratory heterogeneity and, as evidenced by recent molecular studies, genetic heterogeneity.

Hemolysis is the dissolution of red blood corpuscles with liberation of their hemoglobin. Red cells *in vivo* normally undergo dissolution after circulating for approximately 120 days. Hemolysis prior to this is abnormal. Anemia is clinically evident if the rate of red cell destruction is not matched by corresponding production of new cells. Quantitatively, the human red cell is almost entirely hemoglobin [6].

Biochemically, hemoglobin forms only one of three interacting cellular units. The second unit is the cell membrane characterized not only by its lipoprotein structure, but also by its enzymes, e.g., adenosine triphosphatase, and pyridine nucleotidases. The third unit is intracellular and comprises the soluble elements, especially the enzymes, coenzymes, and substrates of glucose metabolism necessary for maintenance of hemoglobin and the membrane. The mature mammalian red cell has no nucleus, no mitochondria or ribosome, no ribonucleic or deoxyribonucleic acid, no Krebs cycle of intermediate metabolism, and no electron carrying system of oxidative phosphorylation. A molecular lesion, genetic or acquired, can occur in any one of the three units, and predispose the cell to premature hemolysis *in vivo*. Induction of hemolysis in most instances requires application of a stress which the predisposed cell, unlike the normal one, cannot withstand. What constitutes a stress depends upon the nature and site of the molecular lesion [7].

In cells with hemoglobin type S, the stress is lowered oxygen tension causing sickling. In cells with membranes altered by sensitizing substances, e.g., the cells of paroxysmal nocturnal

hemoglobinuria, exposure to specific extracellular factors results in hemolysis. In patients whose cells are deficient in glucose-6-phosphate dehydrogenase, ingestion of certain drugs or plant products induces hemolysis. Hemolysis in these individuals is often iatrogenic. Finally, hemolysis may be induced or enhanced by disease, e.g., severe infection [8-11], diabetic acidosis (12, 13), or renal sufficiency (14). Although several molecular lesions of RBC have now been identified and the stresses causing hemolysis are known in many cases, the exact sequence of reactions between the application of stress and resultant hemolysis is not completely known for any hemolytic disorder.

It is well to remember the following factors significant to the biochemistry of hemolysis, but they will not be elaborated in this review: (a) biochemical changes may be shown in red cells of those who are not necessarily susceptible to hemolytic anemia. These changes, not associated with hemolysis, may, instead, represent a genetic disturbance primarily affecting other cells of the body. Their demonstration in the red cells can thus provide evidence of generalized disease. Examples of such changes include galactosemia with deficient galactose-l-phosphate uridyl transferase (15), and diabetes mellitus with increased glutathione reductase (16). (b) Conversely, an enzymatic alteration like glucose-6-phosphate dehydrogenase deficiency which is primarily manifested by susceptibility to hemolysis is not restricted to the red cell but is also found in other tissues of affected individuals (17 to 21). (c) More than one hemolytic order can occur simultaneously in one individual, e.g., thalassemia and glucose-6-phosphate dehydrogenase deficiency (22).

The concept of molecular disease was established when Pauling et *al* (23) discovered that the defect of sickle cell anemia is an altered hemoglobin molecule. Similarly, progress in the biochemistry of hemolysis has been furthered primarily by the discovery of specific defects of erythrocyte metabolism (24). The defects found to date have been in carbohydrate (25) and glutathione (26) metabolism.

In the mature red cell glucose, after conversion to glicose-6P, is catabolized by two pathways: 90% of glucose is metabolized by Emden-Meyerhof (glycolytic) pathway and 10% by the pentose phosphate pathway. The two pathways are connected and the lactic acid may be considered the final product of the glucose metabolism (27-29). It have been suggested that extracellular factors may contribute to the regulation of glucose metabolism and the oxidative process which occur during drug-induced hemolysis have attracted considerable attention. The oxidation of hemoglobin to methemoglobin as a reflection of drug toxicity and a prelytic phenomenon has been known for a long time (30).

The vitality of the red cell is greatest at the moment it enters the circulation and decreases progressively over the next 120 days of its life span. The loss of vitality is accompanied by a decrease in the activity of the pentose-phosphate and glycolytic pathways, presumably because of a gradual decrease in the activity of two key enzymes, G-6-P dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. Consequently, decreases in pyridine nucleotide content, ATP content, and methemoglobin reduction rate ensue and, simultaneously, there is an increase in the cell content of methemoglobin and sodium, and a decrease in cell potassium (31).

A genetic deficiency of the enzyme Glucose-6P dehydrogenase in erythrocytes, therefore, might be regarded in a general way as producing a state of premature senescence and, changes in the hemoglobin molecule of old red cells have been reported (32-34).

Other approaches also offer a great deal to further the investigation of hematological abnormal red cells. The first-generation assays, often used to differentiate red cell membrane disorders from other types of hemolytic anemia, include osmotic fragility, acid glycerol lysis, and autohemolysis tests which measure the extent of or the rate of red cell lysis over a period of time, several minutes to 24 hours, in various incubation media. Typical HS is indicated by an increase of both MCHC and hemoglobin distribution width (as determined by a laser type cell counter), and confirmed by an increased osmotic fragility (35).

Incubated osmotic fragility (OF) testing is considered the gold standard in diagnosing HS in a patient with Coombs-negative spherocytic hemolytic anemia (36), particularly ones of Northern European descent or with a positive family history of undiagnosed anemia. After incubation at 37oC for 24 hours, HS red cells lose membrane surface area more readily than normal cells because their membranes are leaky and unstable. This exposes the membrane defect upon

ISSN: 0974-5335
IJLST (2016), 9(3):28-35

OF testing. When the spleen is present, a subpopulation of fragile erythrocytes that have been conditioned by the spleen form the "tail" of the OF curve that disappears after splenectomy. OF testing suffers from poor sensitivity as ~20% of mild cases of HS are missed after incubation (37).

The main value of the osmotic fragility test, as commonly used in clinical practice, is to confirm important morphological abnormalities of a blood sample, such as the presence of leptocytes and spherocytes (38). However, the osmotic fragility of red cells not only reflects the peculiarities in average membrane and cytoplasm properties of a given sample, but it can also provide information about the distribution of those properties within the sample itself. Thus, by use of density-fractionation techniques, several authors (38-40) have been able to correlate differences in cell density and osmotic fragility with the relative age of the erythrocytes in terms of "young," "mature," and "old" cells. A more systematic and quantitative classification of normal and pathological RBC cells may be obtained by developing a model that relates the osmotic properties of a given blood cell sample with the morphological characteristics of its distribution. When the external osmotic pressure is reduced arbitrarily, the cell volume increases according to a relationship which deviates, to a greater or lesser extent, from ideal behavior. In particular, at the onset of hemolysis, a distribution of critical volumes associated with the distribution of critical osmotic pressures can be expected to hold.

The use of natural products as medicine has increased in all over the world, and popular interest in complementary and alternative medicine (CAM) in Brazil is also as large as it is in other countries (41) especially with regard to phytotherapy, homeopathy and acupuncture. Medicinal plants are used for the human being however several biological effects and the consequences for the health have not been well established yet. Many plants contain active substances that can induce biological effects and their frequent use has been correlated with a high incidence of diseases or undesired biological effect in the population (42-44).

In this review we will be shown the effects of natural products on osmotic fragility of erythrocytes, due to alter the physic-chemical properties of the erythrocyte biomembrane.

METHODOLOGY

Reagents

The reagent NaCl (Merck S.A., Brazil) was used to prepare the solutions to evaluate osmotic fragility. *Hypericum perforatum* was obtained of the *Novo extrato Farmácia homeopática Ltda,* Brazil as a NaCl 0.90% dilution. A commercial *Buzhong Yi Qi Wan* (Gansu Medicines & Health Products Import & Export Corporation) was used in the assays. *Phytic acid* ($C_6H_{18}O_{24}P_6$) and stannous fluoride (SnF_2) were purchased from Sigma Chemicals, USA. *German chamomile* was obtained of the *Hikary Indústria e Comércio LTDA*, Brazil. The leaves of *Lantana camara* were collected in the forest of the city of *Petrópolis*, State of *Rio de Janeiro*, Brazil, and the material collected was identified by the Biologist *Ricardo Carneiro da Cunha Reis*, Botanic Herbarium RB of the *Jardim Botânico*, State of *Rio de Janeiro*, Brazil, where a voucher specimen (4070081) was kept. Heparinized whole blood was withdrawn from male *Wistar* rats (3-4 months age). The osmotic fragility evaluations of the RBC were performed with blood samples incubated with each extract.

The extracts preparation

German chamomile extract was prepared by infusion of one 3.2 mg of German chamomile dust in a total volume of 100 ml of 0.9% NaCl. The solution obtained was considered 0.032mg/mL of German chamomile and denominated chamomile extract.

The extract of *Lantana camara* was prepared with leaves triturated (150 mg, dried in ambient air) added in 15 ml of boiling 0.9% NaCl during 10 minutes. The preparation was filtered through paper (quality filter paper) and considered as 10mg/ml.

As indicated by this manufacturer, lyophilized Buzhong was used to prepare this dried powder. In the preparation of the extract (BYQW), 128 mg of the material was put in a tube with 10ml of saline solution (NaCl 0.9%) that was gently shaken. This suspension was centrifuged in a clinical centrifuge (3000 rpm, 5 min) and the supernatant was considered to be 12.8mg/ml. Dilutions of this solution were performed with 0.9% NaCl solution to obtain diluted solutions.

Others extracts, as well as, Hypericum 6CH and Phytic acid were prepared by dilution in 0.9% NaCl solution to obtain diluted solutions.

The experimental procedures

Blood was withdrawn from a *Wistar* rat cardiac puncture with a heparinized syringe. The osmotic fragility evaluations of the RBC were performed with blood samples incubated with each extract or with sodium chloride solution (0.90% or 0.15 M NaCl) as a control for 60 minutes at room temperature. Briefly, RBC samples (25 µL), treated or not, were gently mixed with hypotonic NaCl (from 0.02 to 0.12M) solutions according to Dacie`s modified method (48b, 49). After 30 min, at room temperature, these tubes were centrifuged (3500 rpm, 15 min).

The supernatants were isolated to determine the hemoglobin optical density (OD) in a spectrophotometer (540 nm). The optical density of each supernatant was compared with that corresponding to stronger hypotonic solution (0.12% or 0.02 M NaCl) that was considered 100% of hemolysis.

The supernatant of the tube, which contained 0.90%, NaCl was considered the "blank tube" for the reaction, because it has no hemolysis. The means and SD of each experiment was determinate and the statistical analysis was performed. The experiments were carried out in compliance with guidelines on the use of live animals in scientific investigations.

Statistical analysis

The results were compared with the control samples, and statistical analysis was performed by independent test ($p<0.05$) to determine the significance of the difference between incubated with extract and control samples.

RESULTS AND DISCUSSION

The osmotic fragility of different extracts of some natural products could be the same of the osmotic fragility of control saline ones. But in the studied extracts isn't not the same. The use of natural products as medicine has increased worldwide, and the interest in complementary and alternative medicine (CAM) is also as large as it is in other countries (41-44) especially with regard to phytotherapy, homeopathy and acupuncture.

Medicinal plants are used for the human being however several biological effects and the consequences for the health have not been well established yet. Many plants contain active substances that can induce biological effects and their frequent use has been correlated with a high incidence of diseases or undesired biological effect in the population (42-43). However, various active compounds derived from medicinal plants have been assessed for their efficacy and tolerability in the treatment of different disorders and have been evaluated in clinical trials (45).

All the results obtained with extracts were summarized on table 1.

In the work with an extract of phytic acid (46) this was unchained, however it (Phytic acid; myo-inositol hexakisphosphate) occurs naturally in many foods derived from plants, mainly in cereals and legumes. In the works with *Matricaria recutita* the osmotic fragility was significantly ($p<0.05$) changed by the presence of the chamomile extract in the isotonic concentrations (47).

The osmotic fragility of extracts of *Hypericum perforatum* was significantly ($p<0.05$) altered by the presence of the hypericin in the isotonic concentrations (48a, 48b), moreover, when an extract of *Lantana camara* was use the significance ($p<0.05$) in the intervals of 0.36 to 0.90% (0.06 to 0.12M) of saline were important, the interval considered between 0.60 (0.10M) to 0.90% of saline is called isotonic (49).

The data obtained from osmotic fragility assay in this work indicated that *Lantana camara* and *Matricaria recutita* extracts could alter the membrane integrity at NaCl concentrations close to physiologic level.

In the same way, the morphological analysis of blood smears suggested alteration on the shape of the red blood cells from whole blood treated with *Lantana camara* extract (49). These alterations on the membrane integrity could be related to components present in the both aqueous extracts capable of interacting with membrane components and that could modify the erythrocyte membrane ions transport or the osmotic transport balance.

It was reported that compounds present in *Lantana camara* alter the function of protein C (50). Other data suggested that an anti-filarial (51) and antitumor (52) effects to this natural product. Moreover, several pharmacological properties have been associated with the studied extracts.

Taken together, these findings could indicate an action of the chemical compounds of these extracts

on membrane structure and they could be in agreement with the results obtained.

Table 1: The variation of Osmotic Fragility on studied extracts.

Plant Extract	Interval concentrations of NaCl (Molar)			Biological Effects suggested	Ref.
	0.10-0.12	0.06-0.10	0.02-0.06		
German chamomile	LI	HI	HI	Could alter the RBC integrity	47
Lantana camara	S	LI	S	Could modify osmotic transport balance or alter the RBC integrity	49
Buzhong	S	HI	HI	Could alter the RBC integrity without altering the shape of the RBC	56
Hypericum perforatum	S	LI	HI	Could alter the RBC morphology altering the ion transport through of the membrane	48b
Phytic acid	S	S	S	The chelating properties of this product were responsible for the effects	46

Ref. (reference); I (increased); S (the same as the control); D (decreased); LI (low increased); HI (high increased); RBC (red blood cell)

The homeopathic *Hypericum perforatum* (Hp) has indicative to depression state (53), and intolerable pains (54). However, we could not find report of natural products diluted and dinamized homeopathicy could interfere on the osmotic resistance of RBC. Erythrocyte osmotic fragility is the resistance of RBC hemolysis to osmotic changes that is used to evaluate RBC friability (55). We had founded that the osmotic fragility of RBC's was changed by presence of Hp homeopathic dose 6CH (HP6CH) in the studied concentrations. The results showed that there was a significant statistical ($p<0.05$) increase in the osmotic fragility of those cells treated with Hp6CH (71%) relative to control (68%) in the interval 2 (0.36 and 0.60% NaCl) that is hypotonic interval of the osmotic curve. In the interval 3 (0.60 and 0.90% NaCl) that is isotonic interval, the osmotic fragility also increased significantly ($p<0.05$) with Hp6CH (16%) relative to control (10%). These experimental data (48b) showed that the osmotic fragility of the RBC can be increased in the presence of Hp6CH solution and we could suggest that this effect may be due to the properties of this medicine that may (i) alter the RBC physical properties, (ii) have a direct or an indirect effect on intracellular sodium ion concentration and (iii) the medicine homeopathic could not be considerate *a placebo effect*.

The treatment with BYQW extract induced significantly ($p<0.05$) the hemolysis at higher concentration used and therefore to increase osmotic fragility of red blood cells when compared to control group (56). The comparison of the means of hemolytic between blood treated with BYQW extract and control was significant ($p<0.05$) to the interval III. BYQW is a traditional magisterial formula composed by different herbs that has been used a long time in the Traditional Chinese Medicine. The action mechanism related with the extract of BYQW is not fully understood (57). These uses in circulatory, immunology and digestive systems have been studied by various occidental and oriental researches. By the way, the distribution mechanism of energy and compounds food, as well as the blood circulations, probably can be altered by BYQW. The result obtained by osmotic fragility assay is in according with the antibacterial effects (58) suggesting that BYQW could act on bacteria cells modifying their membrane integrity.

CONCLUSION:

Our purpose has been to summarize and correlate recent investigations of the physical-chemistry of hemolysis occurring when natural products are used. Erythrocyte metabolic defects involving the action of natural products on the blood system, lead to susceptibility to hemolysis. Study of the defective, compared to normal, erythrocytes allows an integrated approach for the further study of membrane processes, and extracellular factors, as the substances present in the used extracts. It is expected that further investigations, along these lines will advance our understanding of normal cellular metabolism and the mechanism of hemolysis in blood of individuals that used natural products

ACKNOWLEDGMENTS:

We are thanks to CNPq and FAPERJ for the support to this work. We are also thanks to UERJ and UNIFACEX for laboratory, equipment, crew and all material support to this work.

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